The Hæmolytic Action of Sodium Glycocholate.

By Eric Ponder.

(Communicated by Sir E. Sharpey Schafer, F.R.S. Received September 14, 1921.)

(From the Department of Physiology, University of Edinburgh.)

Introduction.

This paper contains a detailed investigation into the action of sodium glycocholate, and into certain phenomena in which this salt plays an important part. The presentation of this research is a matter of some difficulty, since the observations recorded constitute merely the outlines of a very complex subject. It has been thought best, even at the expense of some lack of logical sequence, to present the problems more or less in the order in which they presented themselves for solution, the reader being thus taken over the several questions in the order in which they were investigated. To avoid undue length, no detailed description of methods used is given, if such description is to be found in a previous paper, on the findings of which this work is based (1).

The Physical Condition of Solutions of Bile Salts.

Although there is no definite statement on the subject, the general opinion appears to be that sodium taurocholate and sodium glycocholate form true solutions in water. If this be so, it is remarkable that they possess properties peculiar to colloids. If sodium taurocholate be dissolved in water, a clear yellow solution results. This clearness soon disappears, the solution becomes opalescent and, after about 12 hours, quite opaque. The opalescence is caused, presumably, by the taurocholate passing into a physical state other than that in which it was when first dissolved. The opalescent solution has all the properties of a taurocholate solution; its hæmolytic activity is as great as is that of a clear solution of the same strength. A difference appears on filtering the opalescent solution; the filtrate has a less hæmolytic power than the original solution. This fact, together with the opalescence, suggests that the taurocholate has assumed a less dispersed form.

The more dilute the taurocholate solution is made, the more rapidly does the opalescence appear; in solution in 1 per cent. saline a similar occurrence takes place, but less rapidly than in aqueous solution.

Sodium glycocholate behaves in a similar way, but the appearance of the

opalescence is not so rapid. If in a 1 per cent. solution in saline, the opalescence takes days to appear.

Both salts possess a property peculiar to colloids—they protect a gold sol against precipitation by electrolytes. This protective power is shown in the following way. Using a gold sol prepared by the formaldehyde method, 1 c.c. is precipitated by 0·1 c.c. of 10 per cent. NaCl within 10 seconds. If small quantities of bile salts be added, we find that precipitation is prevented. This protective power is apparent before the solution becomes opalescent, and becomes less as opalescence proceeds, attaining a minimum after about 36 hours. The smallest quantity of taurocholate and glycocholate respectively which will protect 1 c.c. of gold sol against 0·1 c.c. 10 per cent. NaCl is shown in the following Tables, the solutions being kept at 18° C.:—

Table I.

Hours after preparation.	Smallest protecting quantity.	
	Taurocholate (1 in 1000).	Glycocholate (1 in 1000).
	c.c.	c.c.
1 hour	0·2	0·35
12 hours	0·3	0·4
24 „	0·35	0·55
36 „	0·4	0·6
48 „	0·4	0·6

It will be observed that (1) the taurocholate is about twice as protective as the glycocholate, and (2) that, as opalescence becomes established, there is a loss of protective power, the minimum being about half the protective power of the fresh solution.

A more complete study of the physical chemistry relating to this subject is being made.

The Hæmolytic Action of Sodium Glycocholate.

In a paper previously published (1), it has been pointed out that sodium glycocholate behaves in a manner similar to that found in the cases of sodium taurocholate, saponin, and other hæmolytic agents. In dilutions higher than 1 in 1000, the rapidity with which this salt produces hæmolysis depends on the dilution, there being a relatively simple relation between the two. When one examines the action of the salt in concentrations of 1 in 100, 1 in 500, etc., a different behaviour is observed: in three respects especially.

(1) There is great difficulty in obtaining consistent results; this difficulty does not ordinarily exist, for the time required for hæmolysis can, as a rule,

be observed correct to a few seconds, and readily reproduced with consistence. In the case of sodium glycocholate acting in these high concentrations, a variability in the time taken for hæmolysis appears, even when factors such as temperature are controlled.

(2) The salt hæmolyses more rapidly in dilutions of 1 in 1000 and thereabouts, than in dilutions of 1 in 100.

(3) A freshly prepared suspension of red cells seems to be less rapidly hæmolysed by the salt in all dilutions up to 1 in 1000, and especially in dilutions of 1 in 100, and 1 in 50, than is a suspension which is 12 or 18 hours old. This is not unexpected; the envelopes of these old cells being probably weak. The importance of using a freshly prepared suspension for quantitative hæmolytic tests was insisted on in a previous communication.

The following Table gives the times taken for complete hæmolysis of 0·2 c.c. of standard blood suspension, freshly prepared, by various dilutions of this salt, at 18° C.:—

Table II.

δ.	T.	δ.	T.
50	25 minutes.	400	40 minutes.
100	90 „	600	30 „
200	65 „	1000	10 „

It must be understood that this Table is merely representative of the general behaviour of the salt, and that the times given are not the same for all suspensions, but vary with the condition of the cells, etc.

These results may be looked upon as unexpected—hæmolysis occurring more rapidly with a dilution of 1 in 1000 than with a dilution of 1 in 100. It is obvious that the hæmolysis depends on some factor other than the simple solution of the lecithin and cholesterin envelope of the erythrocyte in the solution of bile salt (2). It will further be observed that sodium glycocholate is a feebly hæmolytic agent compared with sodium taurocholate.

It is with occurrences connected with the action of the glycocholate in these relatively high concentrations that this paper is concerned.

It is convenient here to insert a note regarding terminology. The letter T is used to denote the time required for the complete hæmolysis of the amount of blood suspension used. The letter τ represents the temperature at which the experiment is conducted. The symbol δ denotes the number of cubic centimetres which contain 1 grm. of a hæmolytic agent, in a solution which is being used to produce hæmolysis. For detail regarding this nomenclature, the reader is referred to a previous paper (1): in that

paper the special technique employed in these hæmolytic experiments is given in full. In the case of sodium glycocholate, when working at temperatures in the neighbourhood of 20° C., the exact temperature is very important, as in this region a slight rise of temperature greatly accelerates the hæmolysis. Neglect of taking this into account is a fruitful source of error.

The Effect of Blood Serum on the Action of Bile Salts.

Blood serum exerts a powerful inhibitory influence on the hæmolytic action of sodium glycocholate and sodium taurocholate. This is illustrated by the following results, the experiment being carried out in the way indicated in a previous paper (1), and in this case at 18° C. :—

Table III.

	T.
Sodium taurocholate, 1 in 1000	3 minutes.
„ „ 0·1 c.c. serum	16 „
Sodium glycocholate, 1 in 1000	10 „
„ „ 0·1 c.c. serum	37 „

With a view to discovering which constituent of serum produced the inhibition, the inhibitory power, if any, of each constituent of serum was studied. This problem will be dealt with later : the subject for consideration at present being certain phenomena occurring when serum albumin is brought into contact with sodium glycocholate and a blood suspension. The fact that the bile salts cannot exert a hæmolytic action in the presence of blood serum is of great interest, since it throws new light on the controversies regarding hæmolysis and hæmoglobinuria in jaundice (3).

The Effect of Serum Albumin on the Hæmolytic Action of Sodium Glycocholate.

The following solutions are used :—

(1) Solutions of sodium glycocholate in saline (0·95 per cent. NaCl). The strength of these solutions is shown in the following Table :—

Table IV.

Glycocholate.	Final concentration.	Value of δ.
per cent.		
2·5	1 in 100	100
1·25	1 200	200
0·833	1 300	300
0·625	1 400	400
0·5	1 500	500
0·417	1 600	600
0·25	1 1000	1000

The second column gives the concentration of glycocholate in the tube if, of any of these dilutions, 2 c.c. be taken, and have 2 c.c. of saline, or saline solution of albumin, and also 1 c.c. of standard blood suspension, added. The third column gives the value of δ for such a mixture.

In all the experiments recorded below, for convenience, quantities one-fifth of these are used, the final concentrations being the same, *e.g.*, instead of 2 c.c. of glycocholate plus 2 c.c. of saline plus 1 c.c. of suspension, 0·4 c.c. of glycocholate, 0·4 c.c. of saline, and 0·2 c.c. of suspension are used.

(2) A solution of serum albumin in saline. The serum albumin was prepared from blood, dried, and kept for some months before use. The strength of the solution is 1 per cent.

(3) A standard blood suspension, as described in the previous paper (1). This is essentially a 5 per cent. suspension of thrice washed human erythrocytes in normal saline.

If to 0·4 c.c. of 2·5 per cent. glycocholate be added 0·2 c.c. of suspension, and, after an interval of 5 seconds, 0·4 c.c. of serum albumin solution be added, hæmolysis occurs very rapidly, in about 30 seconds. It has been noted above that a 1 per cent. solution of glycocholate takes over an hour to produce hæmolysis of this quantity of suspension. Since, after adding the albumin, the concentration of glycocholate is 1 per cent., and since hæmolysis occurs in about 30 seconds, it is obvious that the serum albumin solution has a powerful accelerating effect on the action of the bile salt. It may be observed that the serum albumin solution is of itself non-hæmolytic: and that the rapid hæmolysis is in no way explained by the fact that the blood cells remain in contact with a 2·5 per cent. solution of glycocholate for 5 seconds, since the salt in this concentration will not produce any hæmolysis in this short time. Control experiments, using saline instead of the serum albumin solution, render the accelerating action of the latter quite clear.

The occurrence of this rapid hæmolysis depends on several factors. The rapid hæmolysis occurs with a mixture of serum albumin, sodium glycocholate, and blood cells. These three substances may, however, be mixed in three different ways:—

Method 1.—Put 0·4 c.c. of glycocholate solution, 2·5 per cent., in a tube, add 0·2 c.c. of blood suspension, and then, after an interval add 0·4 c.c. of serum albumin solution.

Method 2.—Put 0·4 c.c. of serum albumin solution in the tube, add 0·2 c.c. of blood suspension, and after an interval, add 0·4 c.c. of 2·5 per cent. glycocholate solution.

Method 3.—To 0·4 c.c. glycocholate solution add 0·4 c.c. of the serum albumin solution, and then 0·2 c.c. of the blood suspension.

The interval is, for convenience, 5 seconds. In all three cases the composition of the final contents of the tubes is the same. Yet very different results appear.

By method 1, hæmolysis occurs in 30 seconds.

By method 2, hæmolysis occurs in 25 minutes.

By method 3, hæmolysis may occur in a short time as in method 1, or after a long time, as in method 2; the time is usually intermediate between the two.

It is thus obvious that two different phenomena are being observed, according as to whether the glycocholate or the albumin is first brought into contact with the cells. It is further obvious that method 3 is of no use for the giving of consistent results, as the time taken to produce hæmolysis varies under apparently the same set of circumstances. The results given by method (1) will first be considered, as being the more important.

The effects produced by varying the quantity of sodium glycocholate in the above experiment, may now be investigated.

In a series of tubes is placed 0·4 c.c. of varying dilutions of sodium glycocholate, as mentioned above; to each tube is added 0·2 c.c. of blood suspension, and after an interval of 5 seconds, 0·4 c.c. of 1 per cent. serum albumin solution is added. The observation of the 5 seconds interval is very important. The results are expressed in tabular form :—

Table V.

When $\tau = 18$.

δ.	T.	δ.	T.
100	1 minute.	400	55 seconds.
200	2½ minutes.	600	50 ,,
300	1½ ,,	1000	6 minutes.

It will be found that very rapid hæmolysis occurs with all the dilutions of the glycocholate used.

If the same suspension be tested in a similar way after it has stood for a few hours, a different state of affairs will be found; the blood cells have undergone a change on standing. This surprising result occurs with great regularity and, with patience, the stages of the change may be made out. As an example, below is given in tabular form, the behaviour of a freshly prepared standard blood suspension, as time elapsed. The time for hæmolysis of 0·2 c.c. of this suspension by 0·4 c.c. glycocholate of various concentrations plus 0·4 c.c. of 1 per cent. serum albumin, was estimated by method 1, as above, at intervals of 1 hour, 12 hours and 24 hours after preparation, as well as immediately after preparation. The results were as follows :—

Table VI.

When $\tau = 18$.

δ.	Time after preparation.			
	5 minutes.	1 hour.	12 hours.	24 hours.
100	1½ minutes	2½ minutes	18 minutes	40 minutes.
200	2 „	30 „	20 „	15 „
300	1 minute	4½ „	4 „	3 „
400	50 seconds	50 seconds	1 minute	1½ „
600	40 „	50 „	1 „	1½ „
1000	2 minutes	3 minutes	7 minutes	8 „

All blood suspensions exhibit this change, some in greater degree, some in less. The change is not one merely to be detected with care, but a very obvious one, which makes investigation into this subject very difficult, much experience being necessary to correctly interpret results. It will be seen from this Table—which gives a typical result—that the freshly prepared suspension is rapidly hæmolysed by all the concentrations of glycocholate, on the addition of the serum albumin, it may be therefore termed "sensitive." An old suspension, however, is not rapidly hæmolysed except by dilutions of glycocholate in the neighbourhood of 1 in 500; it may, therefore, be called, compared to the fresh suspension, an "insensitive" suspension. This meaning will be attached to these terms in the following pages.

At this point it will be convenient to deal with one essential difference between a sensitive and an insensitive suspension. When a standard blood suspension is prepared, blood is drawn into citrated saline, to prevent coagulation. The suspension is centrifuged, the cells washed thrice with saline, and the cells then added to normal saline (0·95 per cent. NaCl), to form a 5 per cent. suspension. This suspension is normally sensitive. If the blood be drawn into normal saline instead of into citrated saline, and the act of coagulation thus permitted, an insensitive suspension results after washing the cells, and preparing the suspension in the same way as before. This is a constant occurrence; the act of coagulation seems to determine that the suspension shall be insensitive. If the blood be drawn slowly from the finger and clotting thus be allowed to begin, the resulting suspension will be insensitive. This very interesting fact is of use; for, with reasonable care, a sensitive suspension can always be prepared, and also if an insensitive suspension be required, it can with certainty be made.

The changes through which a sensitive suspension goes on standing are very curious; further investigations, to be noted below, throw some light on these changes. It may be observed that there is no difference between a

sensitive and an insensitive suspension as regards the activity of either sodium glycocholate or sodium taurocholate when acting upon it; the difference exists only towards the mixture of glycocholate and albumin. The above mentioned phenomena occur not only with human erythrocytes, but with the red cells of dogs, cats, rabbits and guinea-pigs. The length of time for which a sensitive suspension remains unchanged varies. Some suspensions become insensitive within half an hour of preparation; others remain unchanged for as long as 12 to 20 hours.

Having considered the effect of varying the amount of glycocholate used (Table V) when 0·4 c.c. of 1 per cent. albumin solution is used to accelerate the hæmolysis, it must now be considered what the effect is of varying the amount of serum albumin. The following Table shows this. The substances are mixed in the same way as that adopted for the drawing up of Table V, *i.e.*, by method 1; 0·4 c.c. of albumin is added in each case.

Table VII.

When $\tau = 18$.

Albumin.	Glycocholate.			
	1 in 100.	1 in 400.	1 in 600.	1 in 1000.
2 per cent.	30 seconds	20 seconds	35 seconds	5 minutes.
1 „	28 minutes	30 „	80 „	15 „
0·5 „	43 „	4 minutes	20 „	35 „
0·2 „	56 „	12 „	1½ minutes	17 „

From a consideration of this Table it is obvious that the phenomena are very complex. The subject will be left in the meantime, and referred to again later (Table XIV). It will be sufficient to note here that the quantity of serum albumin used in combination with various dilutions of glycocholate is of the greatest importance. In the above experiment the suspension used was an insensitive one.

Certain of the occurrences met with when the sodium glycocholate, blood cells, and albumin are mixed by method 2 may now be considered.

In this method, 0·4 c.c. of 1 per cent. solution of serum albumin is placed in a tube, 0·2 c.c. of standard suspension is added, and after five seconds, 0·4 c.c. of any desired concentration of sodium glycocholate is added. The results of various dilutions of glycocholate may be given; the suspension used is sensitive.

Table VIII.

When $\tau = 18$

δ.	Method 1.	Method 2.
	T.	T.
100	5 seconds	25 minutes.
200	10 ,,	30 seconds.
300	15 ,,	20 ,,
400	20 ,,	25 ,,
600	25 ,,	40 ,,
1000	2½ minutes	4 minutes.

The hæmolysis does not occur so rapidly with method 2 as with method 1. The difference is most marked when high concentrations of glycocholate are used. In investigating the sensitiveness of a suspension, therefore, it is very important that this difference be kept in mind.

The Protective Action of Serum Albumin.

It has been seen that while serum albumin, if added to a cell suspension in contact with sodium glycocholate, accelerates the hæmolysis produced by the latter, if it be added to a cell suspension it will protect it against the action of the sodium glycocholate and serum albumin mixture.

To investigate this further, one may put up four tubes, as follows:—

Tube 1.—1 c.c. suspension 0·2 c.c. of 1 per cent. serum albumin.

Tube 2.—1 c.c. suspension 0·1 c.c. of 1 per cent. serum albumin.

Tube 3.—1 c.c. suspension 0·05 c.c. of 1 per cent. serum albumin.

Tube 4.—1 c.c. suspension 0·02 c.c. of 1 per cent. serum albumin.

The suspension used is a sensitive one. Allow all tubes to stand for 5 minutes. Examine the suspensions to see if they are sensitive or insensitive. The following result is typical:—

Table IX.

When $\tau = 18$; $\delta = 100$; +0·4 c.c. 1 per cent. albumin.

Tube.	T.	Tube.	T.
Control	15 seconds.	3	5 minutes.
1	40 minutes.	4	1 minute.
2	22 ,,		

The effect, then, of adding a small quantity of serum albumin solution to a sensitive suspension is to render it insensitive. The degree of insensitiveness

produced depends on two factors: (1) the amount of serum albumin added; and (2) the time for which it remains in contact with the cells. A suspension thus made insensitive is not resensitised by washing once in the centrifuge: repeated washings may render it sensitive to some degree.

The Protective Power of Blood Serum.

As serum blood albumin has this effect, it might be supposed that blood serum itself had a similar effect. The following typical experiment shows this clearly:—

Tube 1.—1 c.c. suspension 0·02 c.c. serum.

Tube 2.—1 c.c. suspension 0·015 c.c. serum.

Tube 3.—1 c.c. suspension 0·01 c.c. serum.

Tube 4.—1 c.c. suspension 0·005 c.c. serum.

Allow the tubes to stand 5 minutes. The suspension used is a sensitive one. Test the suspensions to see if they are sensitive or not.

The typical result is as follows:—

Table X.

When $\tau = 18$; $\delta = 100$; +0·4 c.c. 1 per cent. albumin.

Tube.	T.	Tube.	T.
Control	15 seconds.	3	6 minutes.
1	20 minutes.	4	25 „
2	15 „		

The serum thus renders a sensitive suspension insensitive. Such an insensitive suspension is however restored on washing the cells in the centrifuge; an occurrence which is not found in the protection conferred by serum albumin, and suggesting that the protection is conferred in different ways in the two cases. The protection is conferred not by fresh serum only, but by preserved serum, kept for over 18 months by the method of Leers (4).

Animal Experiments.

It is confirmatory of these experiments that the protective action of serum albumin occurs *in vivo* as well as *in vitro*.

If a rabbit be taken and from a vein a small quantity of blood (0·05 c.c.) be drawn into citrated saline, the suspension resulting after washing these cells —preferably once—is sensitive to the mixture of glycocholate and serum albumin. If now 5 c.c. of a 2 per cent. serum albumin solution in saline be injected into a vein, and blood withdrawn from a distant vein about 5 minutes later, the suspension prepared from this blood will be insensitive.

Results for four rabbits may be recorded :—

Table XI.

When $\tau = 18$.

Rabbit.	T, 1st sample.	T, 2nd sample.	Albumin injected.
1	15 seconds	24 minutes	0·1 gramme.
2	20 ,,	15 ,,	0·04 ,,
3	15 ,,	23 ,,	0·08 ,,
4	30 ,,	18 ,,	0·04 ,,

The injection seems to cause no untoward effect on the rabbit, which is under chloroform anæsthesia.

These phenomena will be found to be not peculiar to serum albumin: other similar substances act as powerful accelerators of glycocholate hæmolysis and as protective agents, when used differently, just like serum albumin. Peptone is such a substance: its actions are exactly parallel with those of the albumin. On trying the effects of various animal extracts, similar properties were found to be possessed by both adrenalin and pituitrin (Parke, Davis preparations). Since it has been shown that pituitrin contains histamine, this at once suggests the possibility of the phenomena being due to histamine or histidine, appearing in both the albumin, the peptone, and the pituitrin (5).

The Effect of Histamine on Glycocholate Hæmolysis.

A series of solutions of histamine (Burroughs Wellcome) is prepared, the following dilutions being convenient:—1 in 500, 1 in 1000, 1 in 2000, 1 in 5000, 1 in 8000, and 1 in 10,000. These solutions are made in normal saline (0·95 per cent. sodium chloride).

Histamine and histidine are, *per se*, non-hæmolytic. If 0·4 c.c. of 2·5 per cent. glycocholate solution have 0·2 c.c. of standard blood suspension added, and if, after 5 seconds, 0·4 c.c. of 1 in 1000 histamine be added, hæmolysis occurs instantaneously.

The histamine thus has the accelerating action found with the serum albumin, whose action was probably due to the histamine contained in it as an unavoidable impurity. This is a most interesting fact, for it enables many more exact observations to be made on the phenomena mentioned above in connection with serum albumin. Certain preliminary notes must first be made.

The Effect of Histamine on Colloid Gold.

In view of the fact that sodium glycocholate protects a gold sol, it is important to know the action of histamine on such a colloid. Histamine

precipitates colloid gold, acting powerfully in this respect. Histidine acts in a similar way, but less powerfully. The precipitating action of a 1 in 1000 solution of histamine is very marked, 0·1 c.c. precipitating 1 c.c. of the gold sol used in about 10 seconds.

It is probable, therefore, that histamine acts as a disturber of all colloids, and therefore of sodium glycocholate. We have seen that the hæmolytic action of this substance when in "combination" with serum albumin or histamine is not to be accounted for by simple solubility of the envelope of the erythrocyte in the bile salt: it is possible that surface tension produces the effect, in which case the interaction of a colloid like the glycocholate, and a precipitator of colloids such as histamine, would be of great interest. The consideration that the hæmolysis may be due to changes in the physical state of the solution, connected with occurrences known to colloid chemistry, suggests that the acidity or alkalinity of the hæmolysing solution will be of great importance: the phenomena perhaps being analogous to those of adsorbtion (6).

It will therefore be necessary to investigate (1) the action of serum albumin, and of histamine, on a blood suspension subjected to the hæmolytic action of various amounts of sodium glycocholate; and (2) the effect of acidity or alkalinity on this action.

The following Tables contain such an investigation. The suspension used is an inactive one. The various concentrations of glycocholate are similar to those previously used (Table IV). The quantity of serum albumin added to each tube, in the columns relating to its action, is 0·4 c.c. of a 1 per cent. solution in saline. The quantity of histamine added to each tube, in the columns relating to its action, is 0·4 c.c. of a 1 in 5000 solution in saline.

"Acid histamine," or "acid serum albumin," is made by adding to 10 c.c. of the histamine solution, 1 in 5000, or, to 10 c.c. of the 1 per cent. albumin solution, 0·1 c.c. of decinormal HCl. "Alkaline histamine," or alkaline serum albumin, is prepared by adding to 10 c.c. of either substance in the concentrations mentioned above, 0·1 c.c. of 1 per cent. Na_2CO_3.

The substances were mixed in the order referred to as method 1, *i.e.*, the glycocholate first, then the blood suspension, of which 0·2 c.c. is used, and then, after 5 seconds, the serum albumin or histamine.

Table XII.

When $\tau = 18$.

δ.	Albumin.	Histamine.	Acid albumin.	Alkaline albumin.	Acid histamine.	Alkaline histamine.
100	65 mins.	95 mins.	43 mins.	105 mins.	55 mins.	110 mins.
200	40 „	30 „	3 „	76 „	40 secs.	73 „
300	9 „	90 secs.	30 secs.	58 „	30 „	50 „
400	1 min.	40 „	20 „	35 „	15 „	15 „
500	50 secs.	30 „	30 „	18 „	30 „	4 „
600	1 min.	35 „	50 „	10 „	50 „	45 secs.
1000	5 mins.	3 mins.	3½ mins.	23 „	2½ mins.	1½ mins.

This Table, which is representative of the general results obtained with an inactive suspension, expresses several important points:—

(1) Histamine behaves similarly to the serum albumin, as an accelerator of the glycocholate hæmolysis.

(2) The rendering of the hæmolysing solution acid causes hæmolysis to be more rapid: if the hæmolysing solution be alkaline, the hæmolysis is retarded. The amount of deviation from neutrality is very small in the above case.

(3) The relation between the speed of hæmolysis under the various conditions and the amount of glycocholate used is expressed. Columns 1 and 2 are confirmatory of the Tables illustrating the behaviour of an inactive suspension.

The question of the reaction of the hæmolysing fluid is obviously one of great importance. A series of observations in which the P_H is determined would be ideal: the difficulties attendant upon the use of buffer solutions in connection with these hæmolytic experiments are at present, however, insuperable. The question is being investigated.

It now remains to consider the effect of varying the quantities of histamine employed for accelerating the glycocholate in its hæmolytic action. This is done in the following two Tables, the first Table illustrating the results when a sensitive suspension is used, the second illustrating the results in the case of an insensitive suspension. In each case, the temperature at which the experiments were conducted was 18° C.: the substances were mixed by method 1.

Table XIII.—A. Sensitive Suspension.

Histamine.	Glycocholate.						
1 in	1 in 100.	1 in 200.	1 in 300.	1 in 400.	1 in 500.	1 in 600.	1 in 1000.
500	5 secs.	5 secs.	3 secs.	8 secs.	10 secs.	25 secs.	40 secs.
1,000	7 ,,	7 ,,	5 ,,	15 ,,	20 ,,	35 ,,	1½ mins.
2,000	8 ,,	10 ,,	10 ,,	20 ,,	25 ,,	30 ,,	2 ,,
5,000	45 mins.	15 mins.	30 ,,	30 ,,	28 ,,	20 ,,	1½ ,,
8,000	50 ,,	18 ,,	45 ,,	45 ,,	30 ,,	25 ,,	1 min.
10,000	56 ,,	24 ,,	2½ mins.	2 mins.	1 min.	30 ,,	50 secs.

Table XIV.—B. Insensitive Suspension.

Histamine.	Glycocholate.						
1 in	1 in 100.	1 in 200.	1 in 300.	1 in 400.	1 in 500.	1 in 600.	1 in 1000.
500	5 secs.	5 secs.	5 secs.	8 secs.	10 secs.	15 secs.	40 secs.
1,000	7 ,,	7 ,,	10 ,,	15 ,,	18 ,,	28 ,,	1½ mins.
2,000	8 ,,	10 ,,	15 ,,	20 ,,	25 ,,	30 ,,	2 ,,
5,000	85 mins.	40 mins.	1 min.	30 ,,	25 ,,	23 ,,	1½ ,,
8,000	90 ,,	42 ,,	5 mins.	45 ,,	30 ,,	25 ,,	1 min.
10,000	105 ,,	45 ,,	7 ,,	2 mins.	1 min.	30 ,,	50 secs.

From these somewhat complicated Tables very little new is to be learnt, except that the occurrences which take place when the action of glycocholate of sodium is accelerated by histamine are exceedingly complex. Several points may, however, be noted:—

(1) If these times be plotted on graph paper, a series of curves of definite character will be obtained. The character of the curves, however, does not suggest any generalisation.

(2) The difference between the sensitive and the insensitive suspension may be seen in columns 1; 2 and 3 of the respective Tables. At the other dilutions these differences diminish. This is confirmatory of the observations made with serum albumin.

(3) From an inspection of columns 1 and 2, it appears that there is a very great disproportion between the effect produced by a 1 in 2000 histamine solution and a 1 in 5000 solution. This suggests that the occurrences met with when 1 in 500 to 1 in 2000 histamine is used are different in kind from those met with when more dilute histamine solutions are used, whereas the difference between the activity of the solutions to which the other columns relate is one of degree only. This is very probable, in the light of other considerations, and will be commented on later.

The Protective Action of Histamine.

It has been shown that histamine possesses the accelerating action on the hæmolysis produced by sodium glycocholate, being in this respect similar to the action of the serum albumin solution dealt with in the beginning of the paper. It remains to be shown whether or not it has the protective action of the serum albumin solution (Table IX).

This is a simple matter, in view of the information conveyed in Table XIII. The following experiment illustrates this:—

To 1 c.c. of an active standard blood suspension, add 0·1 c.c. of 1 in 500 histamine. Allow this tube to stand for 5 minutes.

If 0·2 c.c. of this suspension be added to glycocholate and histamine in a tube, it will carry with it a small amount of additional histamine. For instance, if 0·4 c.c. of glycocholate have added 0·2 c.c. of this suspension, and also 0·4 c.c. of 1 in 5000 histamine, the final dilution of histamine in the tube will be 1 in 8330, instead of 1 in 12,500, which it would have been if instead of this suspension containing histamine, a standard suspension had been used. Consulting Table XIII, it will be seen that if 1 in 500 glycocholate be employed for hæmolysis, this slight increase in the histamine concentration is of little consequence, altering the hæmolytic time only by 1 or 2 seconds. Therefore the effect of a dilution of 1 in 500 glycocholate on the standard suspension, before and after it has had histamine added in this quantity, will decide whether or not the histamine has caused a protection of the blood cells.

To two tubes, then, add 0·4 c.c. of a 0·5 per cent. solution of sodium glycocholate; add in the case of one tube, 0·2 c.c. of standard suspension, and in the case of the second, 0·2 c.c. of the suspension containing histamine, as prepared above; after a 5 seconds' interval, add 0·4 c.c. of 1 in 5000 histamine. The tube containing the standard suspension hæmolyses in 30 seconds, that containing the suspension, plus histamine, in 3½ minutes. This demonstrates that the histamine has a protecting effect on the cells against hæmolysis by the glycocholate-histamine system.

If the cells of this suspension plus histamine be washed in saline in the centrifuge, the suspension resulting from adding them to the appropriate amount of saline is still slow in being hæmolysed by these quantities of glycocholate and histamine. This demonstrates that, as in the case of the protection conferred by serum albumin solution, the protection conferred by the histamine is not due merely to the presence of the latter in the saline surrounding the red cells, but due to some change produced in the cells themselves.

The Action of Histidine.

Histidine acts in a manner similar to histamine in accelerating hæmolysis by sodium glycocholate, and, if used in another way, in protecting cells against hæmolysis by histidine and glycocholate. In general, its action in these respects is less marked than that of histamine.

These properties belonging to histamine and to histidine do not appear to be general properties of amino-acids. Glycine and arginin, for instance, do not possess them. A full study of this question is being made, and the point will not be further dealt with in this paper.

Discussion.

It is a much easier matter to observe the phenomena described in this paper than to explain them. A brief discussion of certain points is desirable.

It appears obvious that the explanation of hæmolysis by sodium glycocholate on the grounds that this salt dissolves the envelope of the corpuscle is inadequate, in view of the unusual behaviour of the salt in certain high concentrations, and especially in view of the action of non-hæmolytic substances like histamine when in the presence of a blood suspension and sodium glycocholate. A more probable explanation is one which is based on changes of surface tension; possibly the solvent action of the salt plays a subsequent part. To advance a theory to explain these occurrences is at present impossible; the following suggestion, however, is supported by the majority of the facts, and may be taken as a working hypothesis, useful for the present until further facts are brought to light.

If we consider first the addition of blood cells to a solution of sodium glycocholate, we may say that two occurrences take place: (1) the glycocholate becomes condensed at the interfaces, and therefore on the surface of the red cells; and (2) a solvent action of the glycocholate on the envelope of the cell begins. If histamine, which it has been seen, powerfully disturbs colloid solutions, be added, the colloidal glycocholate probably undergoes a sudden change of physical state, resulting in a sudden variation of the surface tension at the red-cell interfaces, where the glycocholate is collected. This sudden alteration ruptures the cell wall, all the more so as the glycocholate is already attacking the envelope, and therefore is, as it were, continuous with the substances composing it. The rapid hæmolysis produced by the addition of histamine may be thus explained. If, on the other hand, the histamine be added to the cells first, it will not be so condensed at the interfaces as the colloid would be, and will certainly not dissolve the envelope. On the

addition of glycocholate, then, a change of physical state of the latter occurs, with a sudden change of surface tension, as before, but more evenly distributed throughout the fluid, instead of being more marked at the cell interfaces. Hæmolysis will therefore be slower; the possibility that the glycocholate and the histamine may form some species of adsorption compound which has scarcely any hæmolytic action might further enter into the explanation.

Such a consideration is further supported by the fact that the occurrences are so influenced by small changes in reaction; the process of adsorption and similar colloid phenomena being very sensitive in this respect. It may be also noted that the curious results obtained by varying the quantities of the interacting substances point to changes more complex than simple chemical interaction. It appears at times that the phenomena do not occur until certain quantities of the interacting substances are present, *e.g.*, in Tables XIII and XIV, columns 1 and 2. Here possibly the amount of histamine added was insufficient to disturb the glycocholate sufficiently to cause the change of surface tension necessary to produce hæmolysis.

Not the least interesting of these occurrences is the change which seems to occur in the blood cells themselves, both on standing and under the action of histamine. The latter occurrence seems to have no explanation which is even probable at present. The fact that blood cells, prepared in such a way that coagulation is permitted, are insensitive, may be due to some protective action exerted by some product of coagulation.

The whole subject, which may at first sight seem of little practical consequence, is of great importance. The facts show that hæmolysis by simple chemical substances depends on complex factors, and any information which can be gained regarding the true manner of action in these relatively simple cases is of interest in the consideration of the vastly more complex phenomena of hæmolysis by hæmolysins of animal origin.

Summary.

1. Sodium taurocholate and sodium glycocholate are to be considered as colloids. They protect colloid gold against precipitation of electrolytes.

2. Sodium glycocholate is a feebly hæmolytic agent. If histamine or histidine be added to it in suitable proportions, a highly hæmolytic mixture results, although histamine and histidine are of themselves non-hæmolytic. The reaction of the hæmolysing fluid influences the speed of hæmolysis.

3. Histamine, if brought in contact with blood cells, renders them immune to hæmolysis by the histamine glycocholate mixture. Histidine acts similarly. This appears to be due to some change in the cells themselves, and not merely to the presence of the histamine in the fluid.

4. Blood cells which are rapidly hæmolysed by glycocholate and histamine become insensitive on standing, but to a less degree with an old suspension than with a freshly prepared one.

5. Serum albumin and peptone solutions, and also pituitrin, produce both the rapid hæmolysis when mixed with sodium glycocholate, and also the protective effect when added to blood cells. This latter occurs *in vivo* as well as *in vitro*. These occurrences may be due to the presence of histamine or allied substances.

6. Several facts suggest that these phenomena are mainly due to disturbance of surface tension, similar to those which are met with in colloidal solutions. They cannot be explained by the theory that the bile salt dissolves the corpuscle envelope.

7. Suspensions of cells which are derived from blood drawn into a fluid which prevents coagulation behave differently from suspensions of cells which are derived from blood drawn into a fluid which permits coagulation to occur.

8. A protection against hæmolysis by the histamine-glycocholate mixture is also conferred by blood serum.

9. The presence of blood serum inhibits the hæmolytic action of sodium taurocholate and sodium glycocholate.

REFERENCES.

1. Ponder, E., "A Method for investigating the Hæmolytic Activity of Chemical Substances," 'Roy. Soc. Proc.,' B, vol. 92 (1921).
2. Schafer, 'Text-book of Microscopic Anatomy,' p. 370 (1912).
3. Von Noorden, 'Metabolism and Practical Medicine,' vol. 2, p. 260 (1907).
4. Muller, P., 'Serodiagnostic Methods' (Transl. Whitman), p. 20 (1913).
5. Abel and Kubota, 'Jour. Pharm. and Exp. Ther.,' vol. 13, p. 243 (1919).
6. Bayliss, W. M., 'Nature of Enzyme Action,' pp. 22 *et seq.* (1914).